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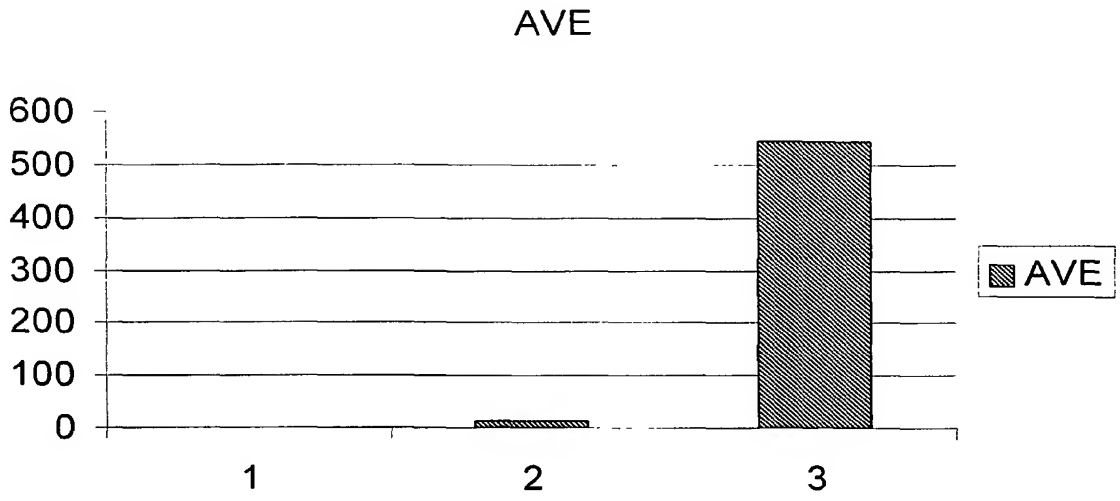
(54) Title: PUTRESCINE-N-METHYLTRANSFERASE PROMOTER



(57) Abstract: Putrescine-N-Methyltransferase promoters, particularly promoters isolated from tobacco, are disclosed, along with recombinant nucleic acids containing the same, expression vectors containing the same, and transgenic plants produced therewith. Methods of use thereof are also disclosed.

WO 02/38588 A2

Putrescine-N-Methyltransferase Promoter

Related Applications

This application claims the benefit of United States Provisional Patent Application Serial No. 60/246,488; Filed November 7, 2000, the disclosure of which is incorporated by reference herein in its entirety.

Field of the Invention

The present invention concerns root-specific promoters useful in plants, along with methods of use thereof, constructs containing the same, and transgenic plants produced with such promoters.

Background of the Invention

A promoter is generally defined as a nucleic acid sequence upsteam or downstream from a transcribed gene, and to which RNA polymerase must bind if it is to transcribe the flanking gene into messenger RNA. A promoter may consist of a number of different regulatory elements that affect a structural gene operationally associated with the promoter in different ways. For example, a regulatory element may enhance or repress expression of an associated structural gene, subject that gene to developmental regulation, or contribute to the tissue-specific regulation of that gene. Modifications to promoters can make possible optional patterns of gene expression, using recombinant DNA procedures. See, e.g., Old and Primrose, Principles of Gene Manipulation (4th Ed., 1989).

Transgenic plants expressing peptides that inhibit or kill a particular pest or pathogen provide a method for decreasing crop damage and loss. For example, expression of the *Bacillus thuringiensis* protein in transgenic corn provides resistance to the European corn borer. However, transgene expression in all tissues of a plant (constitutive expression) can be disadvantageous as it can expose non-target organisms to the transgenic protein and can increase the selective pressure for the

development of pathogens and pests which are resistant to the transgenic protein. High levels of transgene expression throughout a plant may also negatively affect growth and yield of the plant. An alternative strategy is to express a toxic peptide only in the organ or tissue affected by a particular pest or pathogen. Implementation of this strategy against pests and pathogens that attack plant roots has been hampered by the lack of characterized root-specific promoters.

Transcription of a gene is initiated when a stable complex is formed between RNA polymerase enzyme and a gene promoter. Promoters generally occur at the beginning of all transcription units, are typically about 100 base pairs in length, and generally are located immediately upstream from the start site of transcription. See e.g., Maniatis et al., Science 236:1238 (1987). Promoters vary in their "strength"; that is, in their ability to accurately and efficiently initiate transcription. The RNA polymerase holoenzyme is thought to cover a region of about 50 bases immediately upstream of the transcribed region. In some cases the strength of transcription initiation may be enhanced by auxiliary proteins that bind adjacent to the region of the promoter which is immediately upstream from the transcribed DNA. See, e.g., Singer & Berg, Genes and Genomes, 140-145, University Science Books, Mill Valley, Cailf. (1991).

U.S. Patent No. 5,459,252 to Conkling and Yamamoto describes the RB7 root promoter.

U.S. Patent No. 5,837,876 to Conkling, Mendu and Song describes the RD2 root cortex specific promoter, also known as the NtQPT1 promoter.

Summary of the Invention

A first aspect of the present invention is an isolated DNA molecule which directs root specific transcription of a downstream heterologous DNA segment in a plant cell. The promoter is a putrescine-N-methyltransferase (PMT) promoter, such as the tobacco PMT promoter or NtPMT1 promoter. Examples of promoters of the present invention include isolated DNA molecules having a sequence selected from the group consisting of (a) SEQ ID NOs:1-11 provided herein, and (b) DNA sequences which hybridize to any of SEQ ID NOS:1-11 or the complement thereof

(preferably under stringent conditions), and which direct root specific transcription of a downstream heterologous DNA segment in a plant cell.

A further aspect of the present invention is an expression cassette comprising a tobacco PMT promoter and a heterologous DNA segment positioned downstream from, and operatively associated with, the promoter.

A further aspect of the present invention is an expression cassette comprising a root specific promoter and a heterologous DNA segment, the sequence of the root specific promoter as described herein

Further aspects of the present invention are plant cells comprising the above described expression cassettes, methods of making transformed plants from such plant cells, and the transformed plants comprising such transformed plant cells.

Brief Description of the Drawings

Figure 1 shows average GUS expression levels in leaves (column 1), stems (column 2), and roots (column 3) directed by the PMT promoter.

Detailed Description of the Preferred Embodiments

Nucleotide sequences are presented herein by single strand only, in the 5' to 3' direction, from left to right. Nucleotides are represented herein in the manner recommended by the IUPAC-IUB Biochemical Nomenclature Commission.

Specific examples of root specific promoters of the present invention are DNA molecules which have a sequence corresponding to any one of those shown in SEQ ID NOS: 1-11, all of which are discussed in greater detail below. It will be apparent that other sequence fragments from the Tobacco PMT 5' flanking region, longer or shorter than the foregoing sequences, or with minor additions, deletions, or substitutions made thereto, can be prepared which will also carry the TobPMT root specific promoter, all of which are included within the present invention. A further aspect of the present invention includes promoters isolated from other tobacco genes, or from plants other than tobacco as set forth below, which are homologous to the tobacco PMT promoter and are capable of directing root specific transcription of a downstream heterologous DNA segment in a plant cell.

As used herein, a TobPMT promoter refers to a DNA molecule having a sequence identical to, or substantially homologous to, a continuous segment of the DNA found 5' to the transcribed region of the tobacco PMT gene. SEQ ID NO:1 given herein provides the sequence of the region found immediately 5' to the transcription start site in the TobPMT gene. TobPMT promoters include the at least 100 base pair region, the 150 base pair region, or preferably the 200 base pair region immediately 5' to the TobPMT transcribed region, and direct root specific expression. As used herein, regions that are "substantially homologous" are at least 75%, and more preferably are 80%, 85%, 90% or even 95% homologous to the nucleic acid sequence.

As used herein, a root specific promoter is a promoter that preferentially directs expression of an operatively associated DNA, nucleic acid or gene in root tissue, as compared to expression in leaf or stem tissue, or other tissues of the plant.

Root specific promoter sequences from other plants include those which are at least about 75 percent homologous (and more preferably 80%, 85%, 90% or even 95% homologous) to the approximately 100 base segment of the Tobacco PMT promoter immediately upstream of the transcribed DNA region, and which are capable of directing root specific transcription of a downstream heterologous DNA segment in a plant cell. Root specific promoters from other plants include those which are at least about 75 percent homologous (and more preferably 80%, 85%, 90% or even 95% homologous) to the continuous portions of the TobPMT promoter as defined herein by SEQ ID NOS: 1-11, and which are capable of directing root specific transcription of a downstream heterologous DNA segment in a plant cell. Percent homology may be determined by comparing the reference sequence, such as SEQ ID NO: 1 to 11, with another test sequence by the use of a suitable comparison algorithms or by visual inspection. In one embodiment, the substantial identity exists over a region of the sequences that is at least about 20 to 50 residues in length. For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for

the test sequence(s) relative to the reference sequence, based on the designated program parameters. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, Adv. Appl. Math. 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson & Lipman, Proc. Nat'l. Acad. Sci. USA 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by visual inspection (see generally Ausubel et al., supra). One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show relationship and percent sequence identity. It also plots a tree or dendogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, J. Mol. Evol. 35:351-360 (1987). The method used is similar to the method described by Higgins & Sharp, CABIOS 5:151-153 (1989). The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. For example, a reference sequence can be compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps. Another example of algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul et al., J. Mol. Biol. 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying

high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al., supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always>0) and N (penalty score for mismatching residues; always<0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA 89:10915 (1989)).

High stringency hybridization conditions which will permit homologous DNA sequences to hybridize to a DNA sequence as given herein are well known in the art. For example, hybridization of such sequences to DNA disclosed herein may be carried out in 25% formamide, 5X SSC, 5X Denhardt's solution, with 100 ug/ml of single stranded DNA and 5% dextran sulfate at 42° C., with wash conditions of 25% formamide, 5X SSC, 0.1% SDS at 42°. C. for 15 minutes, to allow hybridization of sequences of about 60% homology. More stringent conditions are represented by a wash stringency of 0.3M NaCl, 0.03M sodium citrate, 0.1% SDS at 60° or even 70° C. using a standard in situ hybridization assay. (See Sambrook et al., Molecular Cloning, A Laboratory Manual (2d Ed. 1989)(Cold Spring Harbor Laboratory)). In general, plant DNA sequences which code for root specific promoters and which hybridize to the DNA sequence encoding the tobacco PMT root specific promoters disclosed

herein will be at least 75%, 80%, 85%, 90% or even 95% homologous or more with the sequences of the DNA encoding the tobacco PMT root specific promoters disclosed herein.

Root specific promoters of the present invention are useful in directing tissue specific expression of transgenes in transformed plants. Such tissue-specific transgene expression is useful in providing resistance against damage caused by pests and pathogens which attack plant roots. In addition, as the root is a major sink organ for photosynthate storage, expression of transgenes designed to alter the stored carbohydrates may be directed by such promoters. Exogenous genes of particular interest for root specific expression include those that code for proteins that bind heavy metals (such as metallothionein); proteins that give resistance to soil borne pests and pathogens; proteins that confer resistance to heat, salt (salinity) and drought; proteins for desalinization; and proteins that metabolize plant storage compounds into alternative preferred products or forms.

The root specific promoters of the invention may be used to express proteins or peptides in "molecular farming" applications. Such proteins or peptides include but are not limited to industrial enzymes, antibodies, therapeutic agents, and nutritional products. Such root-specific expression is particularly useful when the plant is a root crop plant such as a sugar beet.

Root specific promoters of the invention are also useful for expressing an oligonucleotide that will decrease or inhibit expression of a native gene in the plant. Such oligonucleotides may be from 4, 6 or 8 nucleotides to 40, 80 or 100 nucleotides in length, or more, and may encode antisense oligonucleotides, ribozymes, sense suppression agents, or other products that inhibit the expression of a native gene.

Tissue specific promoters may also be used to convert pro-pesticides to active forms in selected tissue sites. Hsu et al. Pestic. Sci., 44, 9 (1995) report the use of a chimeric gene comprising the root-specific promoter TobRB7 and the B-glucuronidase enzyme gene, to preferentially convert a pro-pesticide to an active form in roots. The inactive pro-pesticide (a glucuronide of hydroxymethyloxamyl) was applied to foliage and was then transported through plant phloem to roots, where it was converted to an active nematocidal form by glucuronidase.

Additionally, root-cortex specific promoters are useful for histological purposes, to identify or stain root-cortex tissue using a reporter gene such as B-glucurodinase.

The term "operatively associated," as used herein, refers to DNA sequences contained within a single DNA molecule which are associated so that the function of one is affected by the other. Thus, a promoter is operatively associated with a gene when it is capable of affecting the expression of that gene (i.e., the gene is under the transcriptional control of the promoter). The promoter is said to be "upstream" from the gene, which is in turn said to be "downstream" from the promoter.

DNA constructs, or "expression cassettes," of the present invention include, 5'-3' in the direction of transcription, a promoter of the present invention, a heterologous DNA segment operatively associated with the promoter, and, optionally, transcriptional and translational termination regions such as a termination signal and/or a polyadenylation region. These regulatory regions are preferably capable of operating in the transformed cells. The 3' termination region may be derived from the same gene as the transcriptional initiation region or from a different gene.

Plants may be divided into those lacking chlorophyll (such as fungi) and those containing chlorophyll (such as green algae, mosses); and further divided into those containing chlorophyll and having vascular tissue (such as ferns, gymnosperms, conifers, monocots and dicots). The latter group of plants includes those in which roots, stems and leaves may be present. As used herein, the term `plant` encompasses all such organisms described above. As used herein, the term `natural plant DNA` means DNA isolated from non-genetically altered, or untransformed, plants (for example, plant varieties which are produced by selective breeding).

As used herein, the term heterologous gene or heterologous DNA segment means a gene (or DNA segment) which is used to transform a cell by genetic engineering techniques, and which may not occur naturally in the cell. Structural genes are those portions of genes which comprise a DNA segment coding for a protein, polypeptide, or portion thereof, possibly including a ribosome binding site and/or a translational start codon, but lacking a promoter. The term can also refer to copies of a structural gene naturally found within a cell but artificially introduced. Structural genes may encode a protein not normally found in the plant cell in which

the gene is introduced or in combination with the promoter to which it is operationally associated. Genes which may be operationally associated with a promoter of the present invention for expression in a plant species may be derived from a chromosomal gene, cDNA, a synthetic gene, or combinations thereof. As used herein, the term heterologous DNA segment also includes DNA segments coding for non-protein products, such as ribozymes or anti-sense RNAs. Antisense RNAs are well known (see, e.g., U.S. Pat. No. 4,801,540 (Calgene, Inc.)).

Genes of interest for use with the present invention in plants include those affecting a wide variety of phenotypic and non-phenotypic properties. Among the phenotypic properties are proteins, such as enzymes, which provide resistance to various environmental stresses, including but not limited to stress caused by dehydration (resulting from heat, salinity or drought), herbicides, toxic metals, trace elements, pests and pathogens. Resistance may be due to a change in the target site, enhancement of the amount of a target protein in the host cell, increased amounts of one or more enzymes involved with the biosynthetic pathway of a product which protects the host against the stress, and the like. Structural genes may be obtained from prokaryotes or eukaryotes, bacteria, fungi, (e.g., from yeast, viruses, plants, and mammals) or may be synthesized in whole or in part. Illustrative genes include glyphosphate resistant 3-enolpyruvylphosphoshikinate synthase gene, nitrilase, genes in the proline and glutamine biosynthetic pathway, and metallothioneins.

Structural genes operatively associated with the promoter of the present invention may be those which code for a protein toxic to insects, such as *a Bacillus thuringiensis* crystal protein toxic to insects. A DNA sequence encoding a *B. thuringiensis toxin* toxic to Coleoptera, and variations of this sequence wherein the toxicity is retained, is disclosed in U.S. Pat. No. 4,853,331 (see also U.S. Pat. Nos. 4,918,006 and 4,910,136)(the disclosures of all U.S. Patent references cited herein are to be incorporated herein in their entirety by reference). A gene sequence from *B. thuringiensis* which renders plant species toxic to Lepidoptera is disclosed in PCT Application WO 90/02804. PCT Application WO 89/04868 discloses transgenic plants transformed with a vector which promotes the expression of a *B. thuringiensis* crystal protein, the sequence of which may be employed in connection with the present invention. PCT Application WO 90/06999 discloses DNA encoding a *B.*

thuringiensis crystal protein toxin active against Lepidoptera. Another gene sequence encoding an insecticidal crystal protein is disclosed in U.S. Pat. No. 4,918,006. Exemplary of gene sequences encoding other insect toxins are gene sequences encoding a chitinase (e.g., EC-3.2.1.14), as disclosed in U.S. Pat. No. 4,940,840 and PCT Appln. No. WO 90/07001. A gene coding for a nematode-inducible pore protein useful in producing transgenic plants resistant to root nematodes is disclosed in U.S. Pat. Application Ser. No. 08/007,998. Strains of *B. thuringiensis* which produce polypeptide toxins active against nematodes are disclosed in U.S. Pat. Nos. 4,948,734 and 5,093,120.

Where the expression product of the gene is to be located in a cellular compartment other than the cytoplasm, the structural gene may be constructed to include regions which code for particular amino acid sequences which result in translocation of the product to a particular site, such as the cell plasma membrane, or secretion into the periplasmic space or into the external environment of the cell. Various secretory leaders, membrane integration sequences, and translocation sequences for directing the peptide expression product to a particular site are described in the literature. See, for example, Cashmore et al., Biotechnology (1985) 3:803-808, Wickner and Lodish, Science (1985) 230:400-407.

The expression cassette may be provided in a DNA construct which also has at least one replication system. For convenience, it is common to have a replication system functional in *Escherichia coli*, such as ColE1, pSC101, pACYC184, or the like. In this manner, at each stage after each manipulation, the resulting construct may be cloned, sequenced, and the correctness of the manipulation determined. In addition, or in place of the *E. coli* replication system, a broad host range replication system may be employed, such as the replication systems of the P-1 incompatibility plasmids, e.g., pRK290. In addition to the replication system, there may be at least one marker present, which may be useful in one or more hosts, or different markers for individual hosts. That is, one marker may be employed for selection in a prokaryotic host while another marker may be employed for selection in a eukaryotic host, particularly the plant host. The markers may provide protection against a biocide, such as antibiotics, toxins, heavy metals, or the like; may provide complementation by imparting prototrophy to an auxotrophic host; or may provide a

visible phenotype through the production of a novel compound in the plant. Exemplary genes which may be employed include neomycin phosphotransferase (NPTII), hygromycin phosphotransferase (HPT), chloramphenicol acetyltransferase (CAT), nitrilase, and the gentamicin resistance gene. For plant host selection, non-limiting examples of suitable markers are beta-glucuronidase (GUS) (providing indigo production), luciferase (providing visible light production), NPTII (providing kanamycin resistance or G418 resistance), HPT (providing hygromycin resistance), and the mutated aroA gene (providing glyphosate resistance).

The various fragments comprising the various constructs, expression cassettes, markers, and the like may be introduced consecutively by restriction enzyme cleavage of an appropriate replication system and insertion of the particular construct or fragment into the available site. After ligation and cloning, the DNA construct may be isolated for further manipulation. All of these techniques are amply exemplified in the literature. See, e.g., Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1982).

A vector is a replicable DNA construct. Vectors which may be used to transform plant tissue with DNA constructs of the present invention include both Agrobacterium vectors and ballistic vectors, as well as vectors suitable for DNA-mediated transformation. Agrobacterium tumefaciens cells containing a DNA construct of the present invention, wherein the DNA construct comprises a Ti plasmid, are useful in methods of making transformed plants. Plant cells are infected with an Agrobacterium tumefaciens to produce a transformed plant cell, and then a plant is regenerated from the transformed plant cell.

Numerous Agrobacterium vector systems useful in carrying out the present invention are known. For example, U.S. Pat. No. 4,459,355 discloses a method for transforming susceptible plants, including dicots, with an Agrobacterium strain containing the Ti plasmid. The transformation of woody plants with an Agrobacterium vector is disclosed in U.S. Pat. No. 4,795,855. Further, U.S. Pat. No. 4,940,838 to Schilperoort et al. discloses a binary Agrobacterium vector (i.e., one in which the Agrobacterium contains one plasmid having the vir region of a Ti plasmid but no T-DNA region, and a second plasmid having a T-DNA region but no vir region) useful in carrying out the present invention.

Microparticles carrying a DNA construct of the present invention, which microparticle is suitable for the ballistic transformation of a plant cell, are also useful for making transformed plants of the present invention. The microparticle is propelled into a plant cell to produce a transformed plant cell and a plant is regenerated from the transformed plant cell. Any suitable ballistic cell transformation methodology and apparatus can be used in practicing the present invention. Exemplary apparatus and procedures are disclosed in Sanford and Wolf, U.S. Pat. No. 4,945,050, and in Agracetus European Patent Application Publication No. 0 270 356, titled "Pollen-mediated Plant Transformation". When using ballistic transformation procedures, the expression cassette may be incorporated into a plasmid capable of replicating in the cell to be transformed. Examples of microparticles suitable for use in such systems include 1 to 5 .mu.m gold spheres. The DNA construct may be deposited on the microparticle by any suitable technique, such as by precipitation.

A transformed host cell is a cell which has been transformed or transfected with constructs containing a DNA sequence as disclosed herein using recombinant DNA techniques. Plant species may be transformed with the DNA construct of the present invention by the DNA-mediated transformation of plant cell protoplasts and subsequent regeneration of the plant from the transformed protoplasts in accordance with procedures well known in the art.

The promoter sequences disclosed herein may be used to express a heterologous DNA sequence in any plant species capable of utilizing the promoter (i.e., any plant species the RNA polymerase of which binds to the promoter sequences disclosed herein) . Examples of plant species suitable for transformation with the DNA constructs of the present invention include both monocots and dicots, and include but are not limited to tobacco, soybean, potato, cotton, sugarbeet, sunflower, carrot, celery, flax, cabbage and other cruciferous plants, pepper, tomato, citrus trees, bean, strawberry, lettuce, maize, alfalfa, oat, wheat, rice, barley, sorghum and canola. Thus an illustrative category of plants which may be transformed with the DNA constructs of the present invention are the dicots, and a more particular category of plants which may be transformed using the DNA constructs of the present invention are members of the family Solanacae.

Any plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a vector of the present invention. The term "organogenesis," as used herein, means a process by which shoots and roots are developed sequentially from meristematic centers; the term "embryogenesis," as used herein, means a process by which shoots and roots develop together in a concerted fashion (not sequentially), whether from somatic cells or gametes. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristems, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem).

The examples which follow are provided to illustrate various specific embodiments of the present invention, and are not to be construed as limiting the invention.

EXAMPLE 1

Cloning of the *NtPMT* Promoter

Using the *PMT* cDNA sequence published by N. Hibi et al., *Plant Cell* **6,** 723-725 (1994) we designed nested, divergent PCR primers for Inverse-PCR. Tobacco genomic DNA was cut with a variety of restriction endonucleases, ligated at low DNA concentrations (to promote circularization), and then used as a template for Inverse-PCR. The longest amplification product was obtained from genomic DNA that had been digested with *Nde*I. This fragment was cloned and sequenced. Sequence comparisons to the *PMT* cDNA showed sequence identity in the known regions, illustrating that the amplified DNA product was, indeed, the 5' flanking region of the *PMT* gene. The DNA sequence of the *NtPMT* promoter is as follows:

GTATACCAAA AATCAATTCA ACCCCCAAAA CATAATACAA CCAATGTTAA
TGCAATATCT CTGCTGCTAT CACGAAGATA ATTGTAGCTC ACGAAAGTAG
GATACATTAT GTAGGTTACA TCACATAGAG GTAATCTAAA GCTCCCAATA
ATAAGATGTG TAATGTTGAT TATGTAGAAA TTTGCCAGGT TATTTAGAAT
AAACAAGAAG AGGAGAAAAA AAGTACAATT TACCTGAACT CTTGAATGTA
TCCTACAAAT AACCTAGACT TCATGGACGT CAGTTGTCAG TTTACTTTTG

TTTTAATGGT ACATCATTTG TCAAATACTT TATTTGGATA AAAACAGTTT
TGCCTAAGGA GTAAACAGAT CCGGAGTAAG AAAGCAGACG ATTAAAGCAA
TTTTTAAAAA AGGAGAGAGA AATTAATGAG CACACACATA TACTAGTGAA
ATTAGGGTAC TAATTTACTA ATAATTGCAC CGAGACAAAC TTATATTTTA
GTTCCAAAAT GTCAGTCTAA CCCTGCACGT TGTAATAAAT TTTTAACTCT
ATTATATTAT ATCGAGTTGC GCCCTCCACT CCTCGGTGTC CAAATTGTAT
TTAAATGCAT AGATGTTTAA TGGGAGTGTA CAGCAAGCTT TCGGAAAATA
CAAACCATAA TACTTTCTCT TCTTCAATTT GTTTAGTTTA ATTTTGAAAA
TGGAAGTCAT ATCTACCAAC *ACAAATGGCT CTACCATCTT CA*

The sequence corresponding to the PMT cDNA published by Hibi et al. *supra,* is underlined. The initiating ATG is in bold. The sequence used as a primer for inverse-PCR is in italics.

EXAMPLE 2

Transgenic Plants

The *NtPMT1* promoter set forth in Example 1 (SEQ ID NO: 1) was fused to the *GUS* gene in pBI101 and transformed into tobacco in accordance with standard techniques. *See, e.g.,* U.S. Patent No. 5,837,876 at Examples 4-6. Transgenic tobacco was stained for GUS activity using X-Gluc. Transgenic roots stained for GUS activity.

EXAMPLE 3

Deletion Mutants

Additional examples of PMT promoters of the present invention include deletion mutants of the promoter set forth in SEQ ID NO: 1 above. These include mutants with 5' regions deleted, as set forth in SEQ ID NO: 2-6 below, as follows:

SEQ ID NO:2:

AATCAATTCA ACCCCCAAAA CATAATACAA CCAATGTTAA
TGCAATATCT CTGCTGCTAT CACGAAGATA ATTGTAGCTC ACGAAAGTAG
GATACATTAT GTAGGTTACA TCACATAGAG GTAATCTAAA GCTCCCAATA
ATAAGATGTG TAATGTTGAT TATGTAGAAA TTTGCCAGGT TATTTAGAAT
AAACAAGAAG AGGAGAAAAA AAGTACAATT TACCTGAACT CTTGAATGTA
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SEQ ID NO: 3:

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SEQ ID NO: 4:

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SEQ ID NO: 5:

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TTAAATGCAT AGATGTTTAA TGGGAGTGTA CAGCAAGCTT TCGGAAAATA
CAAACCATAA TACTTTCTCT TCTTCAATTT GTTTAGTTTA ATTTTGAAAA
TGGAAGTCAT ATCTACCAAC *ACAAATGGCT CTACCATCTT CA*

SEQ ID NO: 6:

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TCCTACAAAT AACCTAGACT TCATGGACGT CAGTTGTCAG TTTACTTTTG
TTTTAATGGT ACATCATTTG TCAAATACTT TATTTGGATA AAAACAGTTT
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CAAACCATAA TACTTTCTCT TCTTCAATTT GTTTAGTTTA ATTTTGAAAA
TGGAAGTCAT ATCTACCAAC *ACAAATGGCT CTACCATCTT CA*

Additional examples of PMT promoters of the present invention include deletion mutants of the sequence given as SEQ ID NO: 1 above, in which 3' regions are deleted. Examples include SEQ ID NO: 7-9 below:

SEQ ID NO: 7:

GTATACCAAA AATCAATTCA ACCCCCAAAA CATAATACAA CCAATGTTAA
TGCAATATCT CTGCTGCTAT CACGAAGATA ATTGTAGCTC ACGAAAGTAG
GATACATTAT GTAGGTTACA TCACATAGAG GTAATCTAAA GCTCCCAATA
ATAAGATGTG TAATGTTGAT TATGTAGAAA TTTGCCAGGT TATTTAGAAT
AAACAAGAAG AGGAGAAAAA AAGTACAATT TACCTGAACT CTTGAATGTA
TCCTACAAAT AACCTAGACT TCATGGACGT CAGTTGTCAG TTTACTTTTG

TTTTAATGGT ACATCATTTG TCAAATACTT TATTTGGATA AAAACAGTTT
TGCCTAAGGA GTAAACAGAT CCGGAGTAAG AAAGCAGACG ATTAAAGCAA
TTTTTAAAAA AGGAGAGAGA AATTAATGAG CACACACATA TACTAGTGAA
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TTAAATGCAT AGATGTTTAA TGGGAGTGTA CAGCAAGCTT TC

SEQ ID NO: 8:

GTATACCAAA AATCAATTCA ACCCCCAAAA CATAATACAA CCAATGTTAA
TGCAATATCT CTGCTGCTAT CACGAAGATA ATTGTAGCTC ACGAAAGTAG
GATACATTAT GTAGGTTACA TCACATAGAG GTAATCTAAA GCTCCCAATA
ATAAGATGTG TAATGTTGAT TATGTAGAAA TTTGCCAGGT TATTTAGAAT
AAACAAGAAG AGGAGAAAAA AAGTACAATT TACCTGAACT CTTGAATGTA
TCCTACAAAT AACCTAGACT TCATGGACGT CAGTTGTCAG TTTACTTTTG
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TGCCTAAGGA GTAAACAGAT CCGGAGTAAG AAAGCAGACG ATTAAAGCAA
TTTTTAAAAA AGGAGAGAGA AATTAATGAG CACACACATA TACTAGTGAA
ATTAGGGTAC TAATTTACTA ATAATTGCAC CGAGACAAAC TTATATTTTA
GTTCCAAAAT GTCAGTCTAA CCCTGCACGT TGTAATAAAT TTTTAACTCT
ATTATATTAT ATCGAGTTGC GCCCTCCACT CCTCGGTGTC CAAATTGTAT
TTAAATGCAT AGATGTTTAA TGGGAGTGTA

SEQ ID NO: 9:

GTATACCAAA AATCAATTCA ACCCCCAAAA CATAATACAA CCAATGTTAA
TGCAATATCT CTGCTGCTAT CACGAAGATA ATTGTAGCTC ACGAAAGTAG
GATACATTAT GTAGGTTACA TCACATAGAG GTAATCTAAA GCTCCCAATA
ATAAGATGTG TAATGTTGAT TATGTAGAAA TTTGCCAGGT TATTTAGAAT
AAACAAGAAG AGGAGAAAAA AAGTACAATT TACCTGAACT CTTGAATGTA
TCCTACAAAT AACCTAGACT TCATGGACGT CAGTTGTCAG TTTACTTTTG
TTTTAATGGT ACATCATTTG TCAAATACTT TATTTGGATA AAAACAGTTT
TGCCTAAGGA GTAAACAGAT CCGGAGTAAG AAAGCAGACG ATTAAAGCAA
TTTTTAAAAA AGGAGAGAGA AATTAATGAG CACACACATA TACTAGTGAA
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GTTCCAAAAT GTCAGTCTAA CCCTGCACGT TGTAATAAAT TTTTAACTCT
ATTATATTAT ATCGAGTTGC GCCCTCCACT CCTCGGTGTC CAAATTGTAT
TTAAATGCAT AGATGTTTAA

Still additional examples of PMT promoters of the present invention include deletion mutants of the sequence given as SEQ ID NO: 1 above, in which both 3' and 5' regions are deleted. Examples include SEQ ID NO: 10-11 below:

SEQ ID NO: 10:

AATCAATTCA ACCCCCAAAA CATAATACAA CCAATGTTAA
TGCAATATCT CTGCTGCTAT CACGAAGATA ATTGTAGCTC ACGAAAGTAG
GATACATTAT GTAGGTTACA TCACATAGAG GTAATCTAAA GCTCCCAATA
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AAACAAGAAG AGGAGAAAAA AAGTACAATT TACCTGAACT CTTGAATGTA
TCCTACAAAT AACCTAGACT TCATGGACGT CAGTTGTCAG TTTACTTTTG
TTTTAATGGT ACATCATTTG TCAAATACTT TATTTGGATA AAAACAGTTT
TGCCTAAGGA GTAAACAGAT CCGGAGTAAG AAAGCAGACG ATTAAAGCAA
TTTTTAAAAA AGGAGAGAGA AATTAATGAG CACACACATA TACTAGTGAA
ATTAGGGTAC TAATTTACTA ATAATTGCAC CGAGACAAAC TTATATTTTA
GTTCCAAAAT GTCAGTCTAA CCCTGCACGT TGTAATAAAT TTTTAACTCT
ATTATATTAT ATCGAGTTGC GCCCTCCACT CCTCGGTGTC CAAATTGTAT
TTAAATGCAT AGATGTTTAA TGGGAGTGTA CAGCAAGCTT

SEQ ID NO: 11:

ACCCCCAAAA CATAATACAA CCAATGTTAA
TGCAATATCT CTGCTGCTAT CACGAAGATA ATTGTAGCTC ACGAAAGTAG
GATACATTAT GTAGGTTACA TCACATAGAG GTAATCTAAA GCTCCCAATA
ATAAGATGTG TAATGTTGAT TATGTAGAAA TTTGCCAGGT TATTTAGAAT
AAACAAGAAG AGGAGAAAAA AAGTACAATT TACCTGAACT CTTGAATGTA
TCCTACAAAT AACCTAGACT TCATGGACGT CAGTTGTCAG TTTACTTTTG
TTTTAATGGT ACATCATTTG TCAAATACTT TATTTGGATA AAAACAGTTT
TGCCTAAGGA GTAAACAGAT CCGGAGTAAG AAAGCAGACG ATTAAAGCAA
TTTTTAAAAA AGGAGAGAGA AATTAATGAG CACACACATA TACTAGTGAA
ATTAGGGTAC TAATTTACTA ATAATTGCAC CGAGACAAAC TTATATTTTA
GTTCCAAAAT GTCAGTCTAA CCCTGCACGT TGTAATAAAT TTTTAACTCT
ATTATATTAT ATCGAGTTGC GCCCTCCACT CCTCGGTGTC CAAATTGTAT
TTAAATGCAT AGATGTTTAA TGGGAGTGTA

All of the foregoing SEQ ID Nos: 2-11 may be operatively associated with a heterologous nucleic acid or DNA of interest as described above to produce a recombinant nucleic acid which can be inserted into a vector, and in turn into a plant cell and transgenic plants as described above, to cause expression of the heterologous nucleic acid or DNA in the plant.

EXAMPLE 4

Expression Analysis

Figure 1 shows average GUS expression levels in leaves (column 1), stems (column 2), and roots (column 3) directed by the PMT promoter. Data for all 48 independent transformants we examined. There was substantial variation among the GUS levels from transformant to transformant. Important to note is the average GUS activity level (pmol MU/mg protein/min) for roots is 544.82, for stems is 13.24 (~40-fold enhancement in roots), and for leaves is 0.26 (~2000-fold enhancement in roots). Also note that the majority of leaves (44/48) and stems (40/48) had no GUS activity.

The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

THAT WHICH IS CLAIMED IS:

1. An isolated DNA molecule which directs root specific transcription of a downstream heterologous DNA segment in a plant cell, said isolated DNA molecule having a sequence selected from the group consisting of:

(a) SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO: 10 and SEQ ID NO: 11, and

(b) DNA sequences which hybridize to isolated DNA having a sequence of (a) above or the complement of isolated DNA having a sequence of (a) above, under conditions represented by a wash stringency of 0.3M NaCl, 0.03M sodium citrate, 0.1% SDS at 60°, and which direct root specific transcription of a downstream heterologous DNA segment in a plant cell.

2. A DNA construct comprising, in the 5' to 3' direction, a Tobacco PMT promoter and a heterologous DNA segment positioned downstream from said promoter and operatively associated therewith, said promoter directing the root specific expression of said heterologous DNA segment.

3. A DNA construct comprising, in the 5' to 3' direction, a root specific promoter and a heterologous DNA segment downstream from said promoter and operatively associated therewith, wherein said root specific promoter has a sequence selected from the group consisting of:

(a) SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO: 10, and SEQ ID NO: 11, and

(b) DNA sequences which hybridize to the complement of isolated DNA having a sequence of (a) above, under conditions represented by a wash stringency of 0.3M NaCl, 0.03M sodium citrate, 0.1% SDS at 60°, and which direct root specific transcription of a downstream heterologous DNA segment in a plant cell.

4. A DNA construct according to claim 3, wherein said construct is a plasmid.

5. A DNA construct according to claim 3, wherein said heterologous DNA segment is a gene coding for an insecticidal protein.

6. A DNA construct according to claim 4, wherein said heterologous DNA segment is a gene coding for a Bacillus thuringiensis crystal protein toxic to insects.

7. A plant cell transformed with a DNA construct according to claim 3.

8. A method of making a transformed plant, comprising regenerating a plant from a plant cell according to claim 7.

9. A cell containing a DNA construct according to claim 3, and wherein said DNA construct further comprises a Ti plasmid.

10. A method of making a transformed plant, comprising infecting a plant cell with a construct according to claim 9 to produce a transformed plant cell, and then regenerating a plant from said transformed plant cell.

11. A microparticle carrying a DNA construct according to claim 3, wherein said microparticle is suitable for the ballistic transformation of a plant cell.

12. A method of making a transformed plant, comprising propelling a microparticle according to claim 11 into a plant cell to produce a transformed plant cell, and then regenerating a plant from said transformed plant cell.

13. A plant cell protoplast comprising a DNA construct according to claim 3.

14. A method of making a transformed plant, comprising regenerating a plant from a plant cell protoplast according to claim 13.

15. A transformed plant comprising transformed plant cells, said transformed plant cells comprising a heterologous DNA construct, which construct comprises in the 5' to 3' direction, a PMT root specific promoter and a heterologous DNA segment downstream from said promoter and operatively associated therewith, said promoter directing root specific transcription of said heterologous DNA segment.

16. A transformed plant according to claim 15, wherein said promoter has a sequence selected from the group consisting of:

(a) SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO: 10, and SEQ ID NO: 11, and

(b) DNA sequences which hybridize to the complement of isolated DNA having a sequence of (a) above, under conditions represented by a wash stringency of 0.3M NaCl, 0.03M sodium citrate, 0.1% SDS at 60°, and which direct root specific transcription of a downstream heterologous DNA segment in a plant cell.

17. A transformed plant according to claim 15, wherein said plant is a dicot.

18. A transformed plant according to claim 15, wherein said plant is a monocot.

19. A transformed plant according to claim 15, wherein said plant is a tobacco plant.

20. An isolated DNA molecule consisting essentially of a promoter which directs root specific transcription of a downstream heterologous DNA segment in a plant cell and having a sequence selected from the group consisting of SEQ ID NOS:1-11.

21. A DNA construct comprising an expression cassette, which construct comprises, in the 5' to 3' direction, a promoter according to claim 20 and a

heterologous DNA segment downstream from said promoter and operatively associated therewith.

22. A transformed plant comprising transformed plant cells, said transformed plant cells containing a DNA construct according to claim 21.

1/1

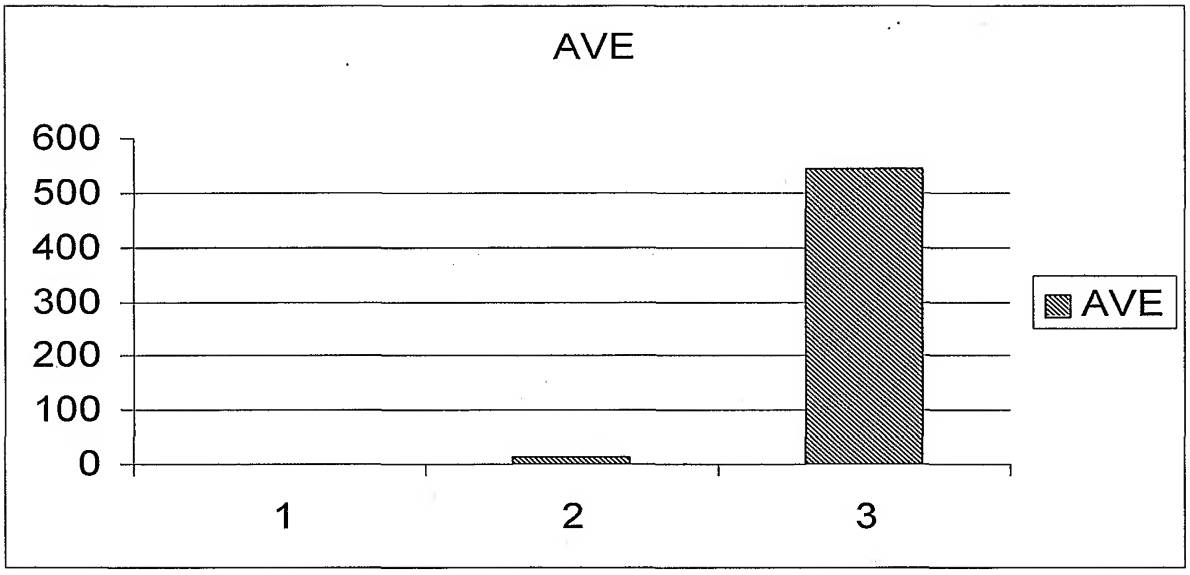

Figure 1

seq530.ST25

SEQUENCE LISTING

<110> Conkling, Mark
Li, Yan

<120> Putrescine-N-Methyltransferase Promoter

<130> 5051-530

<140> To be assigned
<141> 2001-11-06

<150> US 60/246,488
<151> 2000-11-07

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seq530.ST25

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gtaaacagat ccggagtaag aaagcagacg attaaagcaa tttttaaaaa aggagagaga 420
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cgagacaaac ttatatttta gttccaaaat gtcagtctaa ccctgcacgt tgtaataaat 540
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<210> 8
<211> 630
<212> DNA
<213> Deletion mutant of PMT promoter

<400> 8
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tcacatagag gtaatctaaa gctcccaata ataagatgtg taatgttgat tatgtagaaa 180
tttgccaggt tatttagaat aaacaagaag aggagaaaaa aagtacaatt tacctgaact 240
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aattaatgag cacacacata tactagtgaa attagggtac taatttacta ataattgcac 480
cgagacaaac ttatatttta gttccaaaat gtcagtctaa ccctgcacgt tgtaataaat 540
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ttaaatgcat agatgtttaa tgggagtgta 630

<210> 9
<211> 620
<212> DNA
<213> Deletion mutant of PMT promoter

<400> 9
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tcacatagag gtaatctaaa gctcccaata ataagatgtg taatgttgat tatgtagaaa 180
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aattaatgag cacacacata tactagtgaa attagggtac taatttacta ataattgcac 480
cgagacaaac ttatatttta gttccaaaat gtcagtctaa ccctgcacgt tgtaataaat 540
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ttaaatgcat agatgtttaa 620

<210> 10
<211> 630
<212> DNA
<213> Deletion mutant of PMT promoter

<400> 10
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gtaatctaaa gctcccaata ataagatgtg taatgttgat tatgtagaaa tttgccaggt 180

tatttagaat aaacaagaag aggagaaaaa aagtacaatt tacctgaact cttgaatgta 240
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acatcatttg tcaaatactt tatttggata aaaacagttt tgcctaagga gtaaacagat 360
ccggagtaag aaagcagacg attaaagcaa tttttaaaaa aggagagaga aattaatgag 420
cacacacata tactagtgaa attagggtac taatttacta ataattgcac cgagacaaac 480
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<210> 11
<211> 610
<212> DNA
<213> Deletion mutant of PMT promoter

<400> 11
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gttccaaaat gtcagtctaa ccctgcacgt tgtaataaat ttttaactct attatattat 540
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tgggagtgta 610